

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

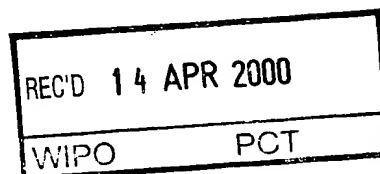
As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.

THIS PAGE BLANK (USPTO)

1E00/26



Patents Office
Government Buildings
Hebron Road
Kilkenny



I HEREBY CERTIFY that annexed hereto is a true copy of documents filed in connection with the following patent application:

Application No. 990157

Date of Filing 26 February, 1999

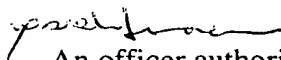
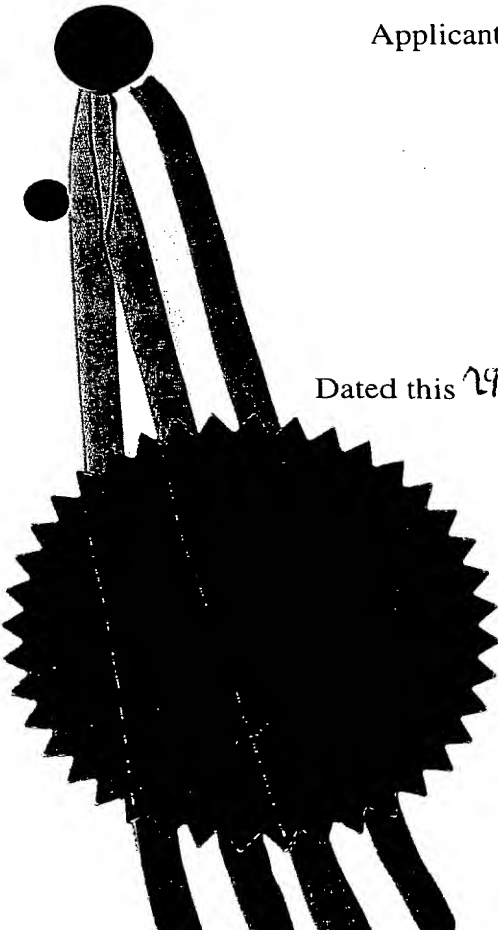
Applicant

UNIVERSITY COLLEGE DUBLIN, a body organised and existing under Charter and being a constituent college of the National University of Ireland of Belfield, Dublin 4, Ireland.

Dated this 29 day of March, 2000.

**PRIORITY
DOCUMENT**

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)



An officer authorised by the
Controller of Patents, Designs and Trademarks.

FP

REQUEST FOR THE GRANT OF A PATENT

PATENTS ACT, 1992

The Applicant(s) named herein hereby request(s)

☒ the grant of a patent under Part II of the Act

☐ the grant of a short-term patent under Part III of the Act
on the basis of the information furnished hereunder.

1. Applicant(s)

Name UNIVERSITY COLLEGE DUBLIN

Belfield,
Dublin 4,
Ireland.

Description/Nationality A body organised and existing under Charter and being a constituent college of the National University of Ireland.

2. Title of Invention

Identification of genes having a role in the presentation of diabetic nephropathy.

3. Declaration of Priority on basis of previously filed application(s) for same invention (Sections 25 & 26)

Previous filing date

Country in or for
which filed

Filing No.

4. Identification of Inventor(s)

Name(s) of person(s) believed
by Applicant(s) to be the inventor(s)

HUGH REDMOND BRADY

CATHERINE MARY GODSON

Address

"Aspen",
Violet Hill,
Killiney,
County Dublin,
Ireland.

12 Yale,
Ardilea,
Dublin 14,
Ireland.

CONTINUED OVER

FINIAN MARY MARTIN

Address

104 Mount Anville Park,
Goatstown,
Dublin 14,
Ireland.

5. Statement of right to be granted a patent (Section 17(2)(b))

The Applicants are assignees of the inventors by virtue of a Deed of Assignment dated February 25, 1999.

6. Items accompanying this Request - tick as appropriate

- (i) ☒ Prescribed filing fee (£100.00)
- (ii) ☒ Specification containing a description and claims
☐ Specification containing a description only
☒ Drawings referred to in description or claims
- (iii) ☒ An abstract
- (iv) ☐ Copy of previous application(s) whose priority is claimed
- (v) ☐ Translation of previous application whose priority is claimed
- (vi) ☐ Authorisation of Agent (this may be given at 8 below if this Request is signed by the Applicant(s))

7. Divisional Application(s)

The following information is applicable to the present application which is made under Section 24 -

Earlier Application No:
Filing Date:

8. Agent

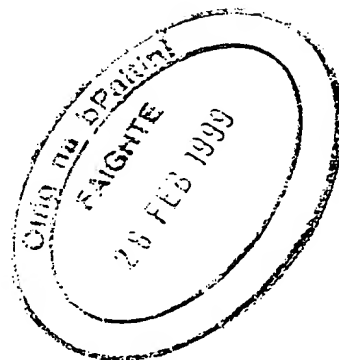
The following is authorised to act as agent in all proceedings connected with the obtaining of a patent to which this request relates and in relation to any patent granted -

Name

Address

ANNE RYAN & CO.

60 Northumberland Road,
Ballsbridge,
Dublin 4,
Ireland.



9. Address for Service (if different from that at 8)

990137

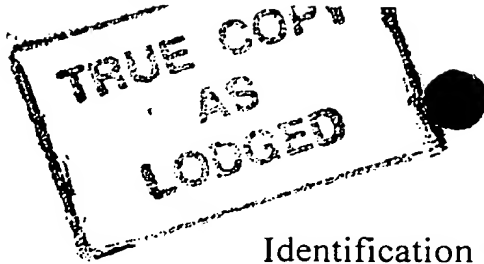
ANNE RYAN & CO., Authorised Patent Agents.

Signed

Name(s): by: Anne Ryan
Capacity (if applicant is a body corporate):

Date

February 26, 1999.



Identification of genes having a role in the presentation of diabetic nephropathy.

5 This invention relates to the characterisation and identification of genes which play a role in diabetes, more particularly in the onset and progression of diabetic nephropathy and to the use of genes so characterised and/or identified as diagnostic markers for diabetic nephropathy and as the basis of drug development programmes.

10 Diabetic nephropathy accounts for over 30 % of end stage renal failure. The pathological hallmark of diabetic nephropathy is glomerulosclerosis due to accumulation of extracellular matrix in the glomerular mesangium. Mesangial matrix accumulation reflects both increased synthesis and decreased degradation of extracellular matrix
15 (ECM) components, and correlates with the clinical onset of proteinuria, hypertension and progressive kidney failure. Hyperglycaemia is a major stimulus for mesangial cell matrix production in diabetic nephropathy. The mechanisms by which hyperglycaemia perturb mesangial cell function are still being appreciated and include direct effects of high
20 extracellular glucose levels and indirect effects transduced through alterations in glomerular haemodynamics and through the actions of advanced glycosylation end products.

25 Propagation of mesangial cells under conditions of high ambient glucose has proved a useful *in vitro* model with which to probe the molecular basis for mesangial matrix accumulation in diabetes, attributable to hyperglycaemia. Specifically, exposure of cultured

mesangial cells to high glucose stimulates *de novo* synthesis of ECM components, such as type IV collagen, fibronectin and laminin, and other products that are accumulated *in vivo* (Ayo, S.H., *et al.* (1990) *Am.J. Pathol.* 136, 1339-1348; Wahab, N.A., *et al.* (1996) *Biochem. J.* 316, 985-992; and Ayo, S.H., *et al.* (1991) *Am. J. Physiol.* 260, F185-F191).

In view of the high morbidity and mortality rate from diabetic nephropathy in diabetics there is a need to identify stimuli which affect the onset and progression of diabetic nephropathy with the aim of preventing such onset or inhibiting or limiting the progression thereof.

The invention provides a method for identifying a gene having a role in the presentation of diabetic nephropathy, which method comprises culturing mesangial cells in the presence of a concentration of glucose sufficient to induce differential expression of a gene susceptible to such differential expression and identifying the gene so induced.

Preferably, the mesangial cells are cultured in the presence of a concentration of glucose sufficient to induce up-regulation of a gene susceptible to such up-regulation.

Further, preferably, the concentration of glucose is greater than 5 mM.

A concentration of 5 mM falls within the normal range of plasma glucose levels in a healthy human subject (4.2 – 6.4 mmol/l).

The concentration of glucose used is suitably in the range 5-30 mM. The concentration of 30 mM was chosen as the classic “*in vitro*” model of diabetic nephropathy which induces changes in mesangial function that mimic human disease. This level is also encountered in many diabetics *in vivo*.

Preferably, the differentially expressed gene is identified by suppression subtractive hybridisation.

Suppression subtractive hybridisation (SSH) is a method based on suppressive PCR that allows creation of subtracted cDNA libraries for the identification of genes differentially expressed in response to an experimental stimulus (Gurskaya, N.G., *et al.* (1996) *Anal. Biochem.* 240, 90-97). SSH differs from earlier subtractive methods by including a normalisation step that equalises for relative abundance of cDNAs within a target population. This modification should enhance the probability of identifying increased expression of low abundance transcripts, and represents a potential advantage over other methods for identifying differentially regulated genes such as differential display-PCR (DD-PCR) (Liang, P., and Pardee, A.B. (1992) *Science* 257, 967-97) and cDNA-representation difference analysis (Hubank, M., and Schatz, D.G., (1994) *Nucleic Acid Res.* 22, 5640-5648).

To date we have used SSH to identify 150 genes differentially induced when human mesangial cells were exposed to high glucose (defined herein as 30 mM versus 5 mM) *in vitro*. These genes included:

5 (a) known regulators of mesangial cell activation in diabetic nephropathy, namely fibronectin, caldesmon, thrombospondin and plasminogen activator inhibitor-1;

10 (b) novel genes; and

(c) genes whose induction by high glucose has not previously been reported as hereinafter described.

15 Prominent among the latter were genes encoding cytoskeleton-associated proteins and connective tissue growth factor (CTGF), a modulator of fibroblast matrix production. We have also demonstrated elevated CTGF mRNA levels in glomeruli of rats with streptozotocin-induced diabetic nephropathy.

20 In one aspect of the invention, the possibility of differential expression due to hyperosmolarity is excluded.

Hyperosmolarity is, however, a component of diabetic nephropathy and thus hyperosmolarity may represent a mechanism
25 through which high glucose induces differential expression of certain genes having a role in the presentation of the disease.

For example, we have shown that mannitol provoked less mesangial cell CTGF expression *in vitro* than high glucose, excluding hyperosmolarity as the key stimulus.

5 High glucose also stimulated expression of transforming growth factor β 1 (TGF- β 1) and addition of TGF- β 1 to mesangial cells triggered CTGF expression. Anti-TGF- β 1 antibody blunted CTGF expression induced by high glucose. Together, these data suggest that (1) high glucose stimulates mesangial CTGF expression by TGF β 1-dependent
10 and independent pathways, and (2) CTGF may be a mediator of TGF- β 1-driven matrix production within a diabetic milieu.

CTGF may therefore be an attractive target for design of novel anti-sclerotic therapies for diabetic glomerulosclerosis.

15 CTGF derived from mesangial cells is a potential stimulus for increased synthesis of ECM proteins and mesangial expansion in diabetic nephropathy. The mechanisms by which high glucose triggers mesangial cell CTGF and, indeed, TGF- β , mRNA expression remain to be defined. Possible upstream triggers of CTGF transcription in
20 response to high glucose include *de novo* synthesis of diacylglycerol (DAG) and subsequent activation of protein kinase C (PKC) (DeRubertis, F.R., and Craven, P., (1994) *Diabetes* 43, 1-8 and Fumo P., *et al.* (1994) *Am. J. Physiol.* 267, F632-F638), non-enzymatic glycation
25 end-products (Brownlee, M., *et al.* (1984) *Ann. Intern. Med* 101, 527-537 and Cohen, M.P., and Ziyadeh, F.N. (1994) *Kidney Int.* 45, 475-484) increased activity of the polyol pathway and disordered

myoinositol metabolism (Goldfarb, S., *et al.* (1991) *Diabetes* 40, 465-471 1991) or through the recruitment of locally generated growth factors such as TGF- β 1 and other mediators. (Sharma K., and Ziyadeh, F.N., (1995) *Diabetes* 44, 1139-1146).

5

TGF- β 1 has been implicated as the key mediator of extracellular matrix accumulation in diabetic nephropathy and other chronic renal disease . Several studies have reported increased expression of TGF- β 1 in renal glomeruli in human and experimental models of diabetes (Park, I., *et al.* (1997) *Diabetes* 46, 473-480; Sharma, K., (1996) *Diabetes* 45, 522-530). Short term administration of TGF- β 1 neutralising antibodies attenuates overexpression of mRNAs encoding matrix components and glomerulosclerosis in the STZ mouse model of diabetes (Park, I., *et al.* (1997) *Diabetes* 46, 473-480). CTGF shares some of the biological actions of TGF- β 1 such as stimulation of cell proliferation and extracellular matrix protein synthesis in fibroblasts. When considered in this context, the results described herein suggest that TGF- β 1 may promote mesangial matrix production, in part, by inducing CTGF synthesis. TGF- β 1 has a complex profile of biological activities that includes pro-inflammatory, pro-fibrotic and anti-inflammatory effects. By targeting CTGF it may be possible to attenuate the sclerosis-inducing effects of TGF- β 1 while preserving its more desirable anti-inflammatory activities.

25 Novel genes identified by the method according to the invention are identified herein as IHG (Induced in *High Glucose*) and DHG

(Down in *High Glucose*) and are represented by genes which include the following sequences:

1) TTGGAATAGTTCTTGCTTTATAAAAATAGTACTGCGATTAAAAAA
 5 AAGCACTTCTGCCAAAGGAACCATGTTCCAACACCGCAAACAAGGTGT
 TCTGCTTAAACAGAGTAAGATACACCACCCCCATCCATCCCTTCCTTC
 CCTGTTCCCCTCCCAACTTGAGTTGTGTTCATTTCGCACCAGTGTCTGG
 GTGGTAGGGATGCTACAGCCACCTAAGGCAAGGAGCCCTGGGAGGTGG
 GAGGGCTTGCATGGTTAAGCACACCAGAACTGAAGCGCAAAAGGGTCA
 10 GCTGTCTTCATCTAGAATCTCTGGATGTTCCCTTCCAGAAAGCATCCCC
 GATGATATCGCAGTGCAAGGGCACTGGCTTTGTCCTGGTCCGGGGTCAC
 TGCCATCTTTTTTTCCTTCCATTTCTGTTGGCAGCTTAATTTCTTTTGT
 CATCACTTCATCCACCTTCTGCCATATCAACACAGTCCCTTTTCCTATA
 CATCGGCAGCTCATTATTATAGTTGATGTTGAATTCAGAAAACAAAAT
 15 CTCATTCTTGTCTGCTGNAAGAGTTCCCTGTAATCTCCCTTGGGCTTG
 TACTGGTGTTAGTCCAGATTGTTG.....
GGTCCTTTAAAGTCTGGTTGC
 TGGGATACACCACGACTCTTCCGGTCAAAGCCTGGGGGATACAGAAGG
 GGCTRGTCCTCAAAGTAATCCCGCCAATAAAACAYATAGCTGGAGGCA
 20 AACTGGGAGGYCACGTGAGTCATGAACTTTACTGGCTCTTCTTTTAAA
 CCAATTGGTTTTTCCGCTTGWACACAAAGCTGTACTCATCACTCTGTCC
 ATAACGCGATCACAATATCCTCTAGTTCTTCCATCACAGTCTGCGCAC
 ATTTGGTCATCAGCTGGAGAGCACGGCTGTCATTGGGTTTTGCAAAGT
 TGTGCTTCTCAGCAAACCGATGGAAATTCCGGCCGTCCAGCCGNACTA
 25 CCACCCAGCAGTGTGCCAGGCAGGTGTCGTCAGCCTCGAAGTCCCTCA
 CGTACTCGAACTTGCTTTTTTGCCATGGTCGCCCCCAATCTCAGGTACC
 GTCTCAGAGTGATGGAAATGGTGGCCAAGGAATCGTGAACCTTAACCTT
 TACAGGCGCCCCACATTCTACACGCGGAAAGGAAAGGGCCAGATAGCC
 CCGCCCCGGAAGTGTTCTCTTCGTGGCTACTCTAGCCGTAGGGCGGTC

ATAGTCTCTCTCGSCTCTCCCTGKAGTTCTTAAMCYYCCAGGGGAAARA
 GGATGGAGGTTT TAGGTT CCTCCGTTAGCACCTTCCACGCTTGCTTCTT
 CCTCCTCCCGGTCTGCGGCAAATCAGTCTCACGAGGTTTTTAAAAATT
 ATTTTTTATCTGCTGGCCTT.....ATGACACAAA
 5 TATTAGGATTTTATTTTTACTATTATCCACCAGCAACAAGATATCAAA
 CACTGGTTCTGTGATTATTTAATGGTGAAAAAGTTGAATAAATCAATT
 TAGTATACCCATATGTTGGAATATTGAGTCCATTTTTCTTTTAAAAAT
 CACACTTTGGAATAATTGATGATACTGGCAAATGCTCAAGCTGAGTGG
 AAAAATATATAAACATTGTATAGGCGAATAATTCCAATCTTGTGCATT
 10 CCCTGTGTAAACCTACATACACAAAAAGAAAAAGACTGAAAGGAACC
 ATCCACAATGCTTTGATCGGGAAAGACGGAGAAACAAAGTGTTAATTT
 TCTTA ACTATAGTTTTNGGTGTATTCCAGATTTTCTACAAGTTAATA
 (IHG-1);

15 2) GCGGCCGCACTCAGCGCCACGCGTCGAAAGCGCAGGCCCCGAGGAC
 CCGCCGCACTGACAGTATGAGCCGCACAGCCTACACGGTGGGAGCCCT
 GCTTCTCCTCTTGGGGACCCTGCTGCCGGCTGCTGAAGGGAAAAAGAA
 AGGGTCCCAAGGTGCCATCCCCCGCCAGACAAGGCCCAGCACAAATGA
 CTCAGAGCAGACTCAGTCGCCCCAGCAGCCTGGCTCCAGGAACCGGGG
 20 GCGGGGGCCAAGGGCGGGGCACTGCCATGCCCGGGGAGGAGGTGCTGGA
 GTCCAGCCAAGAGGCCCTGCATGTGACGGAGCGCAAATACCTGAAGCG
 AGACTGGTGCAAAACCCAGCCGCTTAAGCAGACCATCCACGAGGAAGG
 CTGCAACAGTCGCACCATCATCAACCGCTTCTGTTACGGCCAGTGCAA
 CTCTTTCTACATCCCCAGGCACATCCGGAAGGAGGAAGGTTTCCTTTCA
 25 GTCCTGCTCCTTCTGCAAGCCCAAGAAATTC ACTACCATGATGGTCAC
 ACTCAACTGCCCTGAACTACAGCCACCTACCAAGAAGAAGAGAGTCAC
 ACGTGTGAAGCAGTGTCGTTGCATATCCATCGATTTGGATTAAGCCAA
 ATCCAGGTGCACCCAGCATGTCCTAGGAATGCAGCCCCAGGAAGTCCC
 AGACCTAAAACAACCAGATTCTTACTTGGCTTAAACCTAGAGGCCAGA

AGAACCCCCANCTGCCTCCTGGCAGGAGCCTGCTTGTGCGTAGTTCGT
GTGCATGAGTGTGGATGGGTGCCTGTGGGTGTTTTTAGACACCAGAGA
AAACACAGTCTCTGCTAGAGAGCACTCTCTATTTTTGTAAACATATCT
GCTTTAATGGGGATGTACCAGNAACCCACCT.....AATGAATGT
5 TCATGGAAGAGGCTCCTCTGAGGGCAAGAGACCTGTTTTAGTGCTGCA
TTCGACATGGAAAAGTCCTTTTAACCTGTGCTTGCATCCTCCTTTNCT
CCTCCTNCTCACAANCCATCTCTTCTTAAGTTGATAGTGACTATGGCA
NNCTAATCTCTTGTTTACCAAGGTTCTTAAATTAATTCACTTAACCAT
GATGCAAATGTTTTTCATTTTGTGAAGACCCTCCAGACTCTGGGAGAG
10 GCTGGTGTGGGCAAGGACAAGCAGGATAGTGGAGTGAGAAAGGGAGGG
TGGAGGGTGAGGCCAATCAGGTCCAGSAAAAGTCAGTAGGGACATTG
CAGAAGYTTGAAAGGCCAATACCAGAACACAGGCTGATGCTTCTKAGA
AAGTCTTTTCCTAGTATTTAACAGAACCCAAGTGAACAGAGGAGAAAT
GAGATTGCCAGAAAGTGATTAACCTTTGGCCGTTGCAATCTGCTCAAAC
15 CTAACACCAAACCTGAAAACATAAATACTGACCACTCCTATGTTCCGGAC
CCAAGCAAGTTAGCTAAACCAAACCAACTCCTCTGSYWGTMSCTCAG
GTGGAAAAGAGAGGGTAGTTTAGAACTCTCTGCATAGGGGTGGGAATTA
ATCAAAAACCKCAGAGGCTGAAATTCCTAATACCTTTCTTTATCGTG
GTTATAGTCAGCTCATTTCCATTCCACTATTTCCCATAATGCTTCTGA
20 GAGCCACTAACTTGATTGATAAAGATCCTGCCTCTGCTGAGTGTACCT
GACAGTAAGTCTAAAGATGARAGAGTTTAGGGACTACTCTGTTTTAGC
AAGARATATTKTGGGGGTCTTTTTTGTTTTAACTATTGTCAGGAGATTG
GGCTARAGAGAAGACGACGARAKTWAGGRAATAAAGGGRATTGCCTCT
GGCTAAGARAKTAAGTTAGGTGTTAATACCTGGTAGAAATNTAAGGGA
25 TATGACCTCCCTTTCTTTATGTGCTCACTGAGGATCTGAGGGGACCCT
GTTAGGAGAGCATAGCATCATGATGTATTAGCTGTTTCATCTGCTACTG
GTTGGATGGACATAACTATTGTAACCTATTCAGTATTTACTGGTAGGCA
CTGTCCTCTGATTAACTTGGCCTACTGGCAATGGCTACTTAGGATTG
ATCTAAGGGCCAAAGTGCAGGGTGGGTGAACTTTATTGTACTTTGGAT

TTGGTTAACCTGTTTTCTTCAAGCCTGAGGTTTTATATACAAACTCCC
TGAATACTCTTTTTTGCCTTGTATCTTCTCAGCCTCCTAGCCAAGTCCT
ATGTAATATGGAAAACAAACACTGCAGACTTGAGATTCAGTTGCCGAT
CAAGGCTCTGGCATTTCAGAGAACCCTTGCAACTCGAGAAGCTGTTTTT
5 ATTCGTTTTTTGTTTTGATCCAGTGCTCTCCCATCTAACAATAAACA
GGAGCCATTTCAAGGCGGGAGATATTTTAAACACCCAAAATGGTTGGG
TCTGATTTTCAAACCTTTTAAAACCTTCACTACTGATGATTCTGCACGCT
AAGGCGAATTTGGTCCAAACACATAAGTGTGTGTGTTTTGTATACACT
GTATGACCCCAACCCCAAATCTTTGTATTGTCCACATTCTCCAACAATA
10 AAGCACAGAGTGGATTTAATTAAGCACACAAATGCTAAGGCAGAATTT
TGAGGGTGGGAGAGAAGAAAAGGGAAAGAAGCTGAAAATGTAAAACCA
CACCAGGGAGGAAAAATGACATTCAGAACCAGCAAACACTGAATTTCT
CTTGTTGTTTTAACTCTGCCACAAGAATGCAATTTTCGTTAATGGAGAT
GACTTAAGTTGGCAGCAGTAATCTTCTTTTAGGAGCTTGTACCACAGT
15 CTTGCACATAAGTGCAGATTTGGCTCAAGTAAAGAGAATTTCCCTCAAC
ACTAACTTCACTGGGATAATCAGCAGCGTAACTACCCTAAAAGCATAT
CACTAGCCAAAGAGGGGAAATATCTGTTCTTCTTACTGTGCCTATATTA
AGACTAGTACAAATGTGGTGTGTCTTCCAACTTTCATTGAAAATGCCA
TATCTATACCATATTTTATTTCGAGTCACTGATGATGTAATGATATATT
20 TTTTCATTATTATAGTAGAATATTTTTTATGGCAAGATATTTGTGGTCT
TGATCATACCTATTAAAATAATGCCAAACACCAAATATGAATTTTATG
ATGTACACTTTGTGCTTGGCATTAAAAGAAAAAACACACATCCTGGA
AGTCTGTAAGTTGTTTTTTGTTACTGTAGGTCTTCAAAGTTAAGAGTG
TAAGTGAAAATCTGGAGGAGAGGATAATTTCCACTGTGTGGAATGTG
25 AATAGTTAAATGAAAAGTTATGGTTATTTAATGTAATTATTACTTCAA
ATCCTTTGGTCACTGTGATTTCAAGCATGTTTTCTTTTTCTCCTTTAT
ATGACTTTCTCTGAGTTGGGCAAAGAAGAAGCTGACACACCGTATGTT
GTTAGAGTCTTTTATCTGGTCCAGGGGAACCAAATCTKGACCCAGCT
GAACATGTCTTCCTGAGTCAGTGCCTGAATCTTTATTTTTTAAATTGA

ATGTTTCCTTAAAGGTTAACATTTCTAAAGCAATATTAAGAAAGACTTT
 AAATGTTATTTTGGAGACTTACGATGCATGTATACAAACGAATAGCA
 GATAATGATGACTAGTTCACACATAAAGTCCTTTTAAGGAGAAAATCT
 AAAATGAAAAGTGGATAAACAGAACATTTATAAGTGATCAGTTAATGC
 5 CTAAGAGTGAAAGTAGTTCTATTGACATTCCTCAAGATATTTAATATC
 AACTGCATTATGTATTATGTCTGCTTAAATCATTTAAAAACGGCAAAG
 AATTATATAGACTATGAGGTACCTTGCTGTGTAGGAGGATGAAAGGGG
 AGTTGATAGTCTCATAAACTAATTTGGCTTCAAGTTTCATGAATCTG
 TAACTAGAATTTAATTTTCACCCCAATAATGTTCTATATAGCCTTTGC
 10 TAAAGAGCAACTAATAAATTAAACCTATTCTTTCAAAAAAAAAAAAA
 (IHG-2);

3) AGAAGCAATTTAGGAANCCNACAGNAAANAAATGCTGTTTTATAGG
 AGAGAAAACACGGCACACCAAGGTTAAGTAGTTTGTAGACGATGTTGA
 15 ATAGGTTTCAGGTACAGGTCAATGCAGTGATGAGGAAAGCACCTANGTA
 TACTTGACAGATAGTCCCCCTTGCTTAACACCCCAACTCCTCCACCCTG
 TGCAGTTTNNCTTGTGCCAGTGATCACAGGATTCGCTGAGTGAATTAC
 CATAATTGGATTTAATTCACGAAGGGGATGTTTTTC (IHG-3); and

20 4) ATTGATAGAGGCCCTGTTTCATGACATTTTCATGAGTTTCAATATGT
 TGTTTCAGCATGTTGTGAGGTGACTCTCAGCCCCTTTCCCACTGAGATG
 GACTGTGGTGATGCTGTGAGGGTGTGACTGACACACCTTCATGTGCCC
 AAGCATGGGTTTGATCACAGGTCACATGCAGTTTTTTGGCATAGTAAAT
 GTATCATTGTTCTTTTCCTCCCTCCTAAAGGAAACAGAGGAATCCACC
 25 TGTATGAGAGTGCCATGTAGGGATAAACTTAAAGGACAGATGACACAT
 TGGTCATGTTTCGTGATAAGGAAA (DHG-1).

This invention also provides genes containing sequences 1) – 4) set out above.

The invention also provides use of a gene identified by the method according to the invention:

1) as a diagnostic marker for the progression and presentation of diabetic nephropathy;

2) as an index of disease activity and the rate of progression of diabetic nephropathy; and

3) as a basis for identifying drugs for use in the prevention and/or therapy of diabetic nephropathy.

Thus, it will be appreciated that early diagnosis of diabetic nephropathy based on diagnostic markers identified in accordance with the invention can be used in conjunction with aggressive therapies to prevent full blown development of diabetic nephropathy.

The level of expression of genes identified in accordance with the invention could correlate with the degree of disease progression.

Furthermore, genes identified in accordance with the invention can represent novel therapeutic targets for drug development programmes. Once it has been established that a given gene has a designated role in the pathophysiology of diabetic nephropathy, the

development of new therapeutic agents (such as, for example, small molecules, recombinant inhibitors and receptor antagonists) could be designed to inhibit expression of these genes and, thereby, prevent the development of diabetic nephropathy.

5

Genes identified in accordance with the invention can also be used as a clinical index of progressive renal sclerosis and scarring, as a guide to the response of progressive diabetic nephropathy to therapy and also as markers of the prevention or development thereof.

10

It is possible to generate mouse knock-out (k/o) models for genes identified in accordance with the invention and to generate diabetic k/o mouse models, (for example by treatment with streptozotocin) and determine if onset of diabetic nephropathy is inhibited, reduced or delayed. Thus one can determine if a given knock-out gene has a definite role in the progression and development of diabetic nephropathy.

15

In the accompanying drawings:

20

Fig. 1 is an autoradiograph of CTGF levels analysed by Northern Blot as described in Example 2;

25

Fig. 2 is a graph of the relative amount of CTGF mRNA as estimated by Phosphor Imager quantification as described in Example 2;

Fig. 3 is a 2 % agarose gel showing ethidium-stained PCR products as described in Example 2;

Fig. 4 is a nucleotide sequence alignment of the rat CTGF transcript and the mouse CTGF homologue fisp 12 as described in Example 3;

Fig. 5 is an amino acid sequence alignment of the rat CTGF transcript and the mouse CTGF homologue fisp 12 as described in Example 3;

Fig. 6 is a 2 % agarose gel showing ethidium-stained PCR products as described in Example 3;

Fig. 7 is an autoradiograph of CTGF levels in the presence of TGF- β 1 and TGF- β 1 neutralising antibodies analysed by Northern Blot as described in Example 4;

Fig. 8 is a graph of the relative amount of CTGF mRNA as estimated by Phosphor Imager quantification as described in Example 4;

Fig. 9 is an autoradiograph of CTGF levels in the presence of varying amounts of glucose and TGF- β 1 neutralising antibodies analysed by Northern Blot as described in Example 4;

Fig. 10 is a graph of the relative amount of CTGF mRNA as estimated by Phosphor Imager quantification as described in Example 4;

Fig. 11 is an autoradiograph of CTGF levels in the presence of varying amounts of glucose and PKC inhibitor GF102903X analysed by Northern Blot as described in Example 4; and,

5

Fig. 12 is a graph of the relative amount of CTGF mRNA as estimated by Phosphor Imager quantification as described in Example 4.

The invention will be further illustrated by the following Examples:

10

Example 1

Identification of mesangial cell genes differentially induced by high glucose.

15

a) Cell culture and streptozotocin-induced diabetic rats

Primary human mesangial cells (Clonetics) were cultured as previously reported (Brady, H.R., *et al.* (1992) *Kidney Int.* 42, 480-487 and Denton, M.D., *et al.* (1991) *Am. J. Physiol.* 261, F1071-F1079). Cells (passage 7-11) were maintained in medium containing either 5 mM or 30 mM D-glucose for 7 days. Culture medium was replenished three times during this period to maintain glucose levels in the desired range. To control for the effects of hyperosmolarity, mesangial cells were cultured in media containing 5 mM glucose supplemented with 25 mM mannitol.

20

25

Male Munich-Wistar rats (260-290 g, Simonsen Laboratories) were rendered diabetic by treatment with streptozotocin (STZ; Sigma), 50 g/kg, intravenously as described previously (Zatz, R., *et al.* (1985). *Proc. Natl. Acad. Sci. USA.* 82, 5963-5967). At months 2 and 4 after induction of diabetic nephropathy (DN), rats were anaesthetized with intraperitoneal injection of pentobarbital (50 mg/kg), and the right kidney was excised and weighed immediately. Glomeruli were isolated from renal cortex by the standard sieving method (Brady, H.R. *et al.* (1992) and Denton, M.D. *et al.* (1991) *supra*). Glomerular isolation was completed within 20 minutes of removing the kidney. RNA extraction proceeded immediately thereafter.

b) RNA isolation

Polyadenylated RNA was isolated from mesangial cells using the Microfast Track (Microfast Track is a Trade Mark) kit (Invitrogen). Total RNA was isolated from glomeruli using RNazol solution (TEL-test Inc.).

c) Suppression subtractive hybridisation (SSH)

SSH was performed with the PCR-SELECT cDNA subtraction kit (Clontech) as directed by the manufacturer with the modification that a four-fold greater than recommended amount of driver cDNA was added to the second hybridisation. Starting material consisted of 2 µg of mesangial cell mRNA cultured in 30 mM D-glucose for 7 days as “tester” and 2 µg of mesangial cell mRNA cultured in 5 mM D-glucose

for 7 days as “driver”. Thirty primary PCR cycles and 12 secondary PCR cycles were performed.

d) Cloning and sequencing of cDNAs

5

10

PCR products generated by SSH were subcloned into the PCR 2.1 vector using the original TA cloning kit (Invitrogen). Subcloned cDNAs were isolated by colony PCR amplification. Sequencing was performed using an automated ABI 370A DNA sequencing system. Sequence reactions were carried out with the ABI prism dye terminator cycle sequencing ready reaction kit (Perkin Elmer). The sequences obtained were compared against GenBank/EMBL and Expressed Sequence Tag (EST) databases using BLAST searches.

15

20

SSH analysis suggested differential induction of 16 mRNAs in primary cultures of human mesangial cells propagated for 7 days in 30 mM glucose. Northern Blots performed using formaldehyde denaturation according to standard protocols and quantitated using a Phosphor Imager (Biorad) confirmed differential expression of fifteen of the sixteen subcloned fragments as indicated in Table 1.

In Table 1 ^a refers to the sequence identity based on comparisons with the Genbank/EMBL database;

25

^b refers to an estimate of the size (kb) of the mRNA identified by Northern Blot analysis; and

^c refers to the differential expression of each gene based on Northern Blot analysis of primary human mesangial cells cultured under indicated conditions relative to expression in cells cultured in 5 mM glucose.

Values were obtained by Phosphor-Imaging and were normalised by comparison with GAPDH (*, detected in mesangial cells cultured in 5

mM glucose + 25 mM mannitol and 30 mM glucose, but not in 5 mM

glucose; fold expression is degree of expression relative to that found in 5 mM glucose).

Table 1

Summary of cDNAs identified by SSH as being induced in mesangial cells cultured in high glucose.

5

<u>Gene^a</u>	<u>mRNAkb^b</u>	<u>Differential Expression^c</u>	
		<i>30mM glucose</i>	<i>25mM mannitol+ 5mM glucose</i>
<i>Extracellular Matrix Proteins</i>			
Fibronectin	7.0	2.1-fold	1.5-fold
Thrombospondin	6.0	7.0-fold	8.0-fold
<i>Actin-Binding Proteins</i>			
MRLC	0.9	3.9-fold	1.6-fold
T-plastin	1.2	4.2-fold	1.8-fold
Caldesmon	3.6	3.3-fold	2.8-fold
Profilin	1.0	2.2-fold	2.3-fold
CAP	2.6	1.5-fold	1.7-fold
ARP3	2.5	2.0-fold	1.0-fold
<i>Growth Factors</i>			
CTGF	2.4	3.0-fold	1.5-fold
<i>Others</i>			
PAI-1	2.0	1.2-fold	1.0-fold
	3.0	3.9-fold	2.0-fold
RBM3	1.5	1.8-fold	1.4-fold
Ubiquitin	3.0	2.3-fold	1.7-fold
TCTP	0.8	4.3-fold	2.5-fold
IHG-1	3.4	*	*
IHG-2	2.5	2.0-fold	2.0-fold

Sequence analysis revealed induction of four genes implicated previously in the pathogenesis of diabetic nephropathy: fibronectin, caldesmon, PAI-1, and thrombospondin. Of eleven other cDNA fragments, two encoded novel genes, designated herein as IHG-1 and IHG-2 and nine encoded known genes whose induction by high glucose had not been reported previously. Prominent among the latter genes were connective tissue growth factor (CTGF) and several cytoskeleton-associated proteins, namely profilin, caldesmon, adenyl cyclase-associated protein (CAP), actin-related protein-3 (ARP3), T-plastin, and myosin regulatory light chain (MRLC). Subsequent studies focused on induction of CTGF, a regulator of matrix production in several model systems as described in Examples 2 and 3. The prominence of genes encoding multiple actin-binding proteins is also noteworthy, given recent reports implicating F-actin disassembly in the pathogenesis of mesangial cell dysfunction and glomerular hypertension in diabetic nephropathy (Zhou, X., *et al.* (1995) *Lab. Invest.* 73, 372-383 and Zhou, X. Lai, *et al.* (1997) *Kidney Int.* 51, 1797-1808). The induction of profilin expression is particularly interesting given its role as a regulator of actin polymerization under conditions of cell stress (Sohn, R.H. and Goldschmidt-Clermont, P.J. (1994) *Bioessays* 16, 465-472).

Within a diabetic milieu, high glucose levels may perturb cellular function through glucose-specific actions or by increasing the osmolarity of extracellular fluids. The role of hyperosmolarity as a mediator of gene induction by high glucose was assessed by comparing mRNA levels, as determined by northern blot, in cells cultured in either 30 mM glucose or in 5 mM glucose supplemented with 25 mM

mannitol. High glucose was more effective than high osmolarity at inducing expression of CTGF, myosin regulatory light chain (MRLC), actin related protein 3 (ARP3), T-plastin and translationally controlled tumor protein (TCTP). High glucose and mannitol-induced hyperosmolarity afforded equivalent induction of the other products.

Example 2

CTGF expression in mesangial cells cultured in high glucose.

a) Influence of high ambient glucose on CTGF mRNA levels in human mesangial cells.

CTGF, is a 38kD cysteine-rich secreted peptide known to modulate ECM production in some extrarenal cell types. In Example 1, SSH analysis identified a cDNA fragment of 250 bp which was identical to bases 814-1061 of the human CTGF cDNA. Induction of CTGF mRNA expression in primary human mesangial cells cultured in high glucose was investigated by Northern Blotting as shown in Fig. 1.

In Fig. 1 the lanes represent the following:

Lane 1: RNA from mesangial cells exposed to 5 mM glucose;

Lane 2: RNA from mesangial cells exposed to 5 mM glucose and 25 mM mannitol;

Lane 3: RNA from mesangial cells exposed to 30 mM glucose for seven days.

A 2.4 kb band was detected following hybridisation with the CTGF probe. The relative amounts of CTGF mRNA as estimated by Phosphor Imager quantification are indicated in Fig. 2. All of the values were normalised to GAPDH levels and the results are representative of three independent experiments.

The results indicate that CTGF mRNA expression was between 2.5-3.3-fold higher in mesangial cells cultured in 30 mM glucose as compared with 5 mM glucose.

b) Effect of CTGF on mesangial cell matrix production.

To investigate the direct effects of CTGF up-regulation on matrix production, in particular the effect on collagens I and IV and fibronectin, mesangial cells were incubated with recombinant CTGF protein.

Mesangial cells were serum starved for 24 hr in RPMI 1640 medium supplemented with 0.5 % FBS and then exposed to rhCTGF (8 ng/ml) (a generous gift from Dr. Gary Grotendorst) for 24 hr (Kreisberg, J.I. and Ayo, S.H. (1993). *Kidney Int.* 43, 109-113). Total RNA was extracted and chromosomal DNA was removed using DNase 1 (Gibco-BRL). Equal amounts of cDNA were subsequently amplified by PCR using specific primers for GAPDH (Gen/EMBL accession no.

AJ005371, sense: ACCACAGTCCATGCCATCAC; antisense: TCCACCACCCTGTTGCTGTA), Collagen I (Gen/EMBL accession no. X55525, sense: GGTCTTCCTGGCTTAAAGGG; antisense: GCTGGTCAGCCCTGTAGAAG), Collagen IV (Gen/EMBL accession no. M11315, sense: CCAGGAGTTCCAGGATTTCA; antisense: TTTTGGTCCCAGAAGGACAC) and fibronectin (Gen/EMBL accession no. X02761, sense: CGAAATCACAGCCAGTAG, antisense: ATCACATCCACACGGTAG).

Fig. 3 depicts ethidium-stained panels of a 2% (w/v) agarose gel containing 10 µl of each PCR reaction after electrophoresis.

In Fig. 3 the lanes represent the following:

Lane 1: RT-PCR products from mesangial cells cultured in RPMI 1640 and 0.5% FBS;

Lane 2: RT-PCR products from mesangial cells exposed to rhCTGF (8 ng/ml) for 24 hr.

These results indicate that rhCTGF up-regulates mesangial cell collagens I and IV and fibronectin. These proteins typify matrix accumulation as seen in diabetic nephropathy.

CTGF is a member of a small family of highly homologous proteins termed the CCN family (for CTGF / fisp-12, cef10/cyr61 and Nov) (Bork, P (1993). *FEBS Letts.* 327, 125-130.). These peptides are

characterised by conservation of 38 cysteine residues that constitute more than 10 % of the amino acid content. All members have signal peptides and appear to be secreted via orthodox secretory pathways (Bradham, D.M., (1991) *J. Cell. Biol.* 114, 1285-1294). In the context of diabetic nephropathy, it is intriguing that CTGF which is up-regulated in the presence of ambient glucose, in turn, up-regulates the production of extracellular matrix (ECM). These data demonstrate the potential of CTGF as a stimulus for increased ECM synthesis and mesangial expansion in diabetic nephropathy.

Example 3

Enhanced CTGF expression in renal cortex and isolated glomeruli of rats with STZ-induced diabetic nephropathy.

To assess CTGF expression in diabetic nephropathy *in vivo*, CTGF mRNA levels were measured in RNA isolated from the cortex of rats with STZ-induced diabetes mellitus. To this end, PCR primers for rat CTGF were designed from the sequence of the mouse CTGF homologue, fisp12 (Genbank/EMBL accession no. M70642, sense: CTAAGACCTGTGGAATGGGC; antisense: CTCAAAGATGTCATTGTCCCC) (Ryseck, R.P., (1991) *Cell Growth Differ.* 2, 225-233).

RT-PCR was performed on total RNA extracted from renal cortex of STZ-diabetic rats and age matched controls. The sequence of the rat CTGF transcript was 94 % identical at the nucleotide level (Fig.

4) and 99 % identical at the amino acid level (Fig. 5) to the mouse CTGF homologue fisp12 (bases 783-1123, accession no. M70642). Nucleotides that differ between the two species are given in upper case and the single different amino acid is in bold.

5

Induction of CTGF mRNA was observed in the renal cortex of rats with STZ-induced diabetic nephropathy at four months after administration of STZ, coincident with mesangial expansion and proteinuria as shown in Fig. 6 and data not shown.

10

Fig. 6 depicts ethidium-stained panels of a 2 % (w/v) agarose gel containing 10 µl of each PCR reaction after electrophoresis. CTGF and GAPDH mRNA levels were analysed in total RNA purified from 2 diabetic animals with established nephropathy after four months of diabetes (lanes 1 and 2) and two age matched control animals (lanes 3 and 4).

15

CTGF expression was further localized to glomeruli by RT-PCR analysis of RNA extracted from glomeruli isolated by differential sieving from the renal cortex of rats with STZ-induced diabetic nephropathy. Glomerular levels of CTGF mRNA were increased by 2.5-fold and 1.6-fold after two months and four months of diabetes, respectively, by comparison with age and sex-matched controls. The significance of these observations is further supported by a recent report demonstrating CTGF expression in a screen of human renal diseases including diabetic nephropathy (Ito, Y., *et al.* (1998) *Kidney Int.* 53, 853-861).

20

25

Example 4Induction of mesangial cell CTGF expression by high glucose involves
TGF- β 1 dependent and independent pathways.

5

It has been shown that TGF- β 1 is a stimulus for mesangial matrix accumulation in diabetic nephropathy. In our experimental model as described in Example 1, high glucose concentrations provoked induction of TGF- β 1 mRNA expression in cultured human mesangial cells over the same temporal framework as CTGF expression (data not shown).

10

To assess the role of TGF- β 1 as a stimulus for CTGF expression in response to high glucose, cells were incubated in either 5 mM glucose or 30 mM glucose plus 1 μ l/ml anti-TGF- β 1 antibody for seven days with three changes of medium. Cells were serum starved for 24 hr in RPMI 1640 and 0.5% FBS. 10 ng/ml TGF- β 1 (Calbiochem) or 10 ng/ml TGF- β 1 preadsorbed with 1 μ g/ml neutralising anti-TGF- β 1 polyclonal antibody were subsequently added for 24 hr.

15

20

The role of PKC on CTGF expression in response to high glucose was investigated by culturing the mesangial cells in either 5 mM, 30 mM glucose or 30 mM glucose and the PKC inhibitor GF 102903X.

25

Fig. 7 is an autoradiograph of CTGF mRNA levels analysed by Northern Blot and depicts the results obtained when mesangial cells

were exposed to TGF- β 1 (10 ng/ml) for 24 hr in the presence (lane 3) and absence (lane 2) of anti-TGF- β 1 neutralising antibody (1 μ g/ml). Cells cultured in RPMI 1640 and 0.5 % FBS for 24 hr served as control (lane 1). A 2.4 kb band was detected following hybridisation to the CTGF probe. The blot was stripped and reprobed with GAPDH. The relative amount of CTGF mRNA as estimated by Phosphor Imager quantification (Fig. 8). Values were normalised to GAPDH levels and the results are representative of two independent experiments.

These results indicate that TGF- β 1 is a potent inducer of increased CTGF mRNA levels under these conditions. This effect was inhibited by the addition of a neutralising anti-TGF- β 1 antibody as depicted in Fig. 7.

Fig. 9 is an autoradiograph of CTGF mRNA levels analysed by Northern Blot and depicts the results obtained when mesangial cells were exposed to 5 mM glucose (lane 1), 30 mM glucose (lane 2) and 30 mM glucose in the presence of anti-TGF- β 1 neutralising antibodies (1 μ g/ml) (lane 3) for seven days. A 2.4 kb band was detected following hybridisation to the CTGF probe. The blot was stripped and probed with GAPDH. The relative amount of CTGF mRNA as estimated by Phosphor Imager quantification (Fig. 10). Values were normalised to GAPDH levels.

The neutralising anti-TGF- β 1 antibody partially attenuated the glucose-induced increase in CTGF transcript level in mesangial cells grown in 30 mM glucose for 7 days (Fig. 9), suggesting that high

glucose triggers mesangial cell CTGF expression through TGF- β 1-dependent and independent pathways.

Fig. 11 is an autoradiograph of CTGF mRNA levels analysed by Northern Blot and depicts the results obtained when mesangial cells were exposed to 5 mM glucose (lane 1), 30 mM glucose (lane 2) and 30 mM glucose in PKC inhibitor GF102903X (10 μ M) (lane 3) for four days. A 2.4 kb band was detected following hybridisation to the CTGF probe. The blot was stripped and probed with GAPDH. The relative amount of CTGF mRNA as estimated by Phosphor Imager quantification (Fig. 12). Values were normalised to GAPDH levels.

Whereas the PKC inhibitor GF102903X was without effect on TGF- β 1-induced CTGF expression in our system (data not shown), this compound afforded partial inhibition of high glucose-induced CTGF expression (Fig. 11).

CTGF shares some of the biological actions of TGF- β 1 such as stimulation of cell proliferation and extracellular matrix protein synthesis in fibroblasts. When considered in this context, our results suggest that TGF- β 1 may promote mesangial matrix production, in part, by inducing CTGF synthesis. TGF- β 1 has a complex profile of biological activities that includes pro-inflammatory, pro-fibrotic and anti-inflammatory effects. By targeting CTGF it may be possible to attenuate the sclerosis-inducing effects of TGF- β 1 while preserving its more desirable anti-inflammatory activities.

Example 5Further characterisation of IHG-2

5 IHG-2 is a mesangial cell gene which we have identified as
being induced in human mesangial cells by high extracellular glucose as
described in Example 1. To further characterise this gene, IHG-2 was
searched against the dbEST using the BLAST algorithm. This search
identified a clone that was 94 % identical to ESTAA071138, clone no:
10 530117 3'. The sequence for the 5' end of this clone was also in the
database, which again identified multiple ESTs. These ESTs showed
homology with the 3' untranslated region (UTR) of a rat cDNA clone
known as drm/Gremlin. Gremlin/drm, together with DAN and cerberus,
are members of the cystine knot superfamily which includes TGF β and
15 bone morphogenetic protein (BMP). A second EST W48852, clone
no:324951 3', was identified from the IHG-2 BLAST. The 5' end of this
clone, EST W48619, was also searched against the database, from
which EST AA373348 was obtained. This clone showed homology with
the drm 3' UTR, approximately 500 bp from the open reading frame
20 (ORF). Thus, it was possible to make a direct link from IHG-2 to within
500 bp of the ORF of drm/gremlin. Therefore, by establishing a link
between EST AA37348 and the ORF of drm/gremlin, we can confirm
that IHG-2 is part of the 3' UTR of this gene. Primers were designed to
recognise the ORF, IHG-2, and the EST clone AA373348. An initial
25 PCR using primers corresponding to the start site of the gremlin/drm
gene together with a primer within the IHG-2 clone would give a
predicted product of approximately 2.5 kb. This product was nested

with primers corresponding to the 3' end of the ORF of gremlin and the EST clone AA373348, generating a product of approximately 500 bp, thus verifying that this EST is in the UTR of the human *drm/gremlin* gene. Therefore, IHG-2 is part of the *drm/gremlin* gene.

Claims: -

1. A method for identifying a gene having a role in the presentation of diabetic nephropathy, which method comprises culturing mesangial cells in the presence of a concentration of glucose sufficient to induce differential expression of a gene susceptible to such differential expression and identifying the gene so induced.
2. A method according to Claim 1, wherein the mesangial cells are cultured in the presence of a concentration of glucose sufficient to induce up-regulation of a gene susceptible to such up-regulation.
3. A method according to Claim 1 or 2, wherein the concentration of glucose is greater than 5 mM.
4. A method according to any preceding claim, wherein the differentially expressed gene is identified by suppression subtractive hybridisation.
5. A method according to any one of Claims 1-4, wherein the possibility of differential expression due to hyperosmolarity is excluded.
6. A method according to any one of Claims 1-5, wherein the gene so differentially expressed is a gene which includes a sequence selected from:

1) TTGGAATAGTTCTTGCTTTATAAAAATAGTACTGCGATTAA
 AAAAAAAGCACTTCTGCCAAAGGAACCATGTTCCAACACCGCAAACAA

GGTGTCTGCTTAAACAGAGTAAGATACACCACCCCATCCATCCCTT
CCTTCCCTGTTCCCCTCCCAACTTGAGTTGTGTCATTCGCACCAGTGT
CCTGGGTGGTAGGGATGCTACAGCCACCTAAGGCAAGGAGCCCTGGGA
GGTGGGAGGGCTTGCATGGTTAAGCACACCAGAACTGAAGCGCAAAAG
5 GGTCAGCTGTCTTCATCTAGAATCTCTGGATGTTCCCTCCAGAAAGCA
TCCCCGATGATATCGCAGTGCAAGGGCACTGGCTTTGTCCTGGTCCGG
GTCACTGCCATCTTTTTTCCTTCCATTTCTGTTGGCAGCTTAATTTCT
TTTGTCATCACTTCATCCACCTTCTGCCATATCAACACAGTCCCTTTC
CTATACATCGGCAGCTCATTATTATAGTTGATGTTGAATTCAGAAAAC
10 AAAATCTCATTCTTGTCTGCTGNAAGAGTTCCTGTAATCTCCCTTGG
GCTTGTACTGGTGTAGTCCAGATTGTTG.....
.....GGTCCTTTAAAGTCTG
GTTGCTGGGATACACCACGACTCTTCCGGTCAAAGCCTGGGGGATACA
GAAGGGGCTRGTCCTCAAAGTAATCCCGCCAATAAAACAYATAGCTGG
15 AGGCAAACCTGGGAGGYCACGTGAGTCATGAACCTTACTGGCTCTTCTT
TTAAACCAATTGGTTTTCCGCTTGWACACAAAGCTGTACTCATCACTC
TGTCCATAACGCGATCACAATATCCTCTAGTTCTTCCATCACAGTCTG
CGCACATTTGGTCATCAGCTGGAGAGCACGGCTGTCATTGGGTTTTGC
AAAGTTGTGCTTCTCAGCAAACCGATGGAAATTCCGGCCGTCCAGCCG
20 NACTACCACCCAGCAGTGTGCCAGGCAGGTGTCGTCAGCCTCGAAGTC
CCTCACGTACTCGAACTTGCTTTTTGCCATGGTCGCCCCCAATCTCAG
GTACCGTCTCAGAGTGATGGAAATGGTGGCCAAGGAATCGTGAACCTT
AACTTTACAGGCGCCCCACATTCTACACGCGGAAAGGAAAGGGCCAGA
TAGCCCCGCCCCGGAAGTGTTCTCTTCGTGGCTACTCTAGCCGTAGGG
25 CGGTCATAGTCTCTCTCGSCTCTCCCTGKAGTTCTTAAMCYCCAGGG
AAARAGGATGGAGGTTTAGGTTCCCTCCGTTAGCACCTTCCACGCTTGC
TTCTTCCTCCTCCCGGTCTGCGGCAAATCAGTCTCACGAGGTTTTTAA
AAATTATTTTTTATCTGCTGGCCTT.....ATGAC
ACAAATATTAGGATTTTATTTTTACTATTATCCACCAGCAACAAGATA

TCAAACACTGGTTCTGTGATTATTTAATGGTGAAAAAGTTGAATAAAT
 CAATTTAGTATACCCATATGTTGGAATATTGAGTCCATTTTTCTTTTA
 AAAATCACACTTTGGAATAATTGATGATACTGGCAAATGCTCAAGCTG
 AGTGGAAAAATATATAAACATTGTATAGGCGAATAATTCCAATCTTGT
 5 GCATTCCCTGTGTAAACCTACATACACAAAAAGAAAAAGACTGAAAG
 GAACCATCCACAATGCTTTGATCGGGAAAGACGGAGAAACAAAGTGTT
 AATTTTCTTAACTATAGTTTTNGGTGTATTCCAGATTTTCTACAAGTT
 AATA;

10 2) GCGGCCGCACTCAGCGCCACGCGTCGAAAGCGCAGGCCCCG
 AGGACCCGCGCACTGACAGTATGAGCCGCACAGCCTACACGGTGGGA
 GCCCTGCTTCTCCTCTTGGGGACCCTGCTGCCGGCTGCTGAAGGGAAA
 AAGAAAGGGTCCCAAGGTGCCATCCCCCGCCAGACAAGGCCCAGCAC
 AATGACTCAGAGCAGACTCAGTCGCCCCAGCAGCCTGGCTCCAGGAAC
 15 CGGGGGCGGGGCCAAGGGCGGGGCACTGCCATGCCCGGGGAGGAGGTG
 CTGGAGTCCAGCCAAGAGGCCCTGCATGTGACGGAGCGCAAATACCTG
 AAGCGAGACTGGTGCAAACCCAGCCGCTTAAGCAGACCATCCACGAG
 GAAGGCTGCAACAGTCGCACCATCATCAACCGCTTCTGTTACGGCCAG
 TGCAACTCTTTCTACATCCCCAGGCACATCCGGAAGGAGGAAGGTTCC
 20 TTTCAGTCCTGCTCCTTCTGCAAGCCCAAGAAATTCACCTACCATGATG
 GTCACACTCAACTGCCCTGAACTACAGCCACCTACCAAGAAGAAGAGA
 GTCACACGTGTGAAGCAGTGTCGTTGCATATCCATCGATTTGGATTAA
 GCCAAATCCAGGTGCACCCAGCATGTCCTAGGAATGCAGCCCCAGGAA
 GTCCCAGACCTAAAACAACCAGATTCTTACTTGGCTTAAACCTAGAGG
 25 CCAGAAGAACCCCCANCTGCCTCCTGGCAGGAGCCTGCTTGTGCGTAG
 TTCGTGTGCATGAGTGTGGATGGGTGCCTGTGGGTGTTTTTAGACACC
 AGAGAAAACACAGTCTCTGCTAGAGAGCACTCTCTATTTTTGTAAACA
 TATCTGCTTTAATGGGGATGTACCAGNAACCCACCT.....AATG
 AATGTTTCATGGAAGAGGCTCCTCTGAGGGCAAGAGACCTGTTTTAGTG

CTGCATTTCGACATGGAAAAGTCCTTTTAACTGTGCTTGCATCCTCCT
TTNCTCCTCCTNCTCACAANCCATCTCTTCTTAAGTTGATAGTGACTA
TGGCANNCTAATCTCTTGTTTACCAAGGTTCTTAAATTAATTCACCTA
ACCATGATGCAAATGTTTTTTCATTTTGTGAAGACCCTCCAGACTCTGG
5 GAGAGGCTGGTGTGGGCAAGGACAAGCAGGATAGTGAGTGAGAAAGG
GAGGGTGGAGGGTGAGGCCAATCAGGTCCAGSAAAAGTCAGTAGGGA
CATTGCAGAAGYTTGAAAGGCCAATACCAGAACACAGGCTGATGCTTC
TKAGAAAGTCTTTTCCTAGTATTTAACAGAACCCAAGTGAACAGAGGA
GAAATGAGATTGCCAGAAAGTGATTAACCTTTGGCCGTTGCAATCTGCT
10 CAAACCTAACACCAAACCTGAAAACATAAATACTGACCACTCCTATGTT
CGGACCCAAGCAAGTTAGCTAAACCAAACCAACTCCTCTGSYWYGMS
CTCAGGTGGAAAAGAGAGGTAGTTTAGAACTCTCTGCATAGGGGTGGG
AATTAATCAAAAACCKCAGAGGCTGAAATTCCTAATACCTTTTCCTTTA
TCGTGGTTATAGTCAGCTCATTTCCATTCCACTATTTCCCATAATGCT
15 TCTGAGAGCCACTAACTTGATTGATAAAGATCCTGCCTCTGCTGAGTG
TACCTGACAGTAAGTCTAAAGATGARAGAGTTTAGGGACTACTCTGTT
TTAGCAAGARATATTKTGGGGGTCTTTTTGTTTTAACTATTGTCAGGA
GATTGGGCTARAGAGAAGACGACGARAKTWAGGRAATAAAGGGRATTG
CCTCTGGCTAAGARAKTAAGTTAGGTGTTAATACCTGGTAGAAATNTA
20 AGGGATATGACCTCCCTTTCTTTATGTGCTCACTGAGGATCTGAGGGG
ACCCTGTTAGGAGAGCATAGCATCATGATGTATTAGCTGTTTCATCTGC
TACTGGTTGGATGGACATAACTATTGTAACCTATTAGTATTTACTGGT
AGGCACTGTCCTCTGATTAACTTGGCCTACTGGCAATGGCTACTTAG
GATTGATCTAAGGGCCAAAGTGCAGGGTGGGTGAACTTTATTGTACTT
25 TGGATTTGGTTAACCTGTTTTCTTCAAGCCTGAGGTTTTATATACAAA
CTCCCTGAATACTCTTTTTGCCTTGTATCTTCTCAGCCTCCTAGCCAA
GTCCTATGTAATATGGAAAACAAACACTGCAGACTTGAGATTCAGTTG
CCGATCAAGGCTCTGGCATTTCAGAGAACCCTTGCAACTCGAGAAGCTG
TTTTTATTTTCGTTTTTGTTTTGATCCAGTGCTCTCCCATCTAACAACCT

AAACAGGAGCCATTTCAAGGCGGGAGATATTTTAAACACCCAAAATGG
TTGGGTCTGATTTTCAAACCTTTTAAACTTCACTACTGATGATTCTGC
ACGCTAAGGCGAATTTGGTCCAAACACATAAGTGTGTGTGTTTTGTAT
ACACTGTATGACCCCAACCCCAAATCTTTGTATTGTCCACATTCTCCAA
5 CAATAAAGCACAGAGTGGATTTAATTAAGCACACAAATGCTAAGGCAG
AATTTTGAGGGTGGGAGAGAAGAAAAGGGAAAGAAGCTGAAAATGTAA
AACCACACCAGGGAGGAAAAATGACATTCAGAACCAGCAAACACTGAA
TTTCTCTTGTTGTTTTAACTCTGCCACAAGAATGCAATTTTCGTTAATG
GAGATGACTTAAGTTGGCAGCAGTAATCTTCTTTTAGGAGCTTGTACC
10 ACAGTCTTGCACATAAGTGCAGATTTGGCTCAAGTAAAGAGAATTTCC
TCAACACTAACTTCACTGGGATAATCAGCAGCGTAACTACCCTAAAAG
CATATCACTAGCCAAAGAGGGAAATATCTGTTCTTCTTACTGTGCCTA
TATTAAGACTAGTACAAATGTGGTGTGTCTTCCAACCTTCATTGAAAA
TGCCATATCTATAACCATATTTTATTTCGAGTCACTGATGATGTAATGAT
15 ATATTTTTTTCATTATTATAGTAGAATATTTTTTATGGCAAGATATTTGT
GGTCTTGATCATACCTATTAAAATAATGCCAAACACCAAATATGAATT
TTATGATGTACACTTTGTGCTTGGCATTAAAAGAAAAAACACACATC
CTGGAAGTCTGTAAGTTGTTTTTTGTTACTGTAGGTCTTCAAAGTTAA
GAGTGTAAGTGAAAAATCTGGAGGAGAGGATAATTTCCACTGTGTGGA
20 ATGTGAATAGTTAAATGAAAAGTTATGGTTATTTAATGTAATTATTAC
TTCAAATCCTTTGGTCACTGTGATTTCAAGCATGTTTTCTTTTTCTCC
TTTATATGACTTTCTCTGAGTTGGGCAAAGAAGAAGCTGACACACCGT
ATGTTGTTAGAGTCTTTTATCTGGTCCAGGGGAACCAAATCTKGACC
CAGCTGAACATGTCTTCCTGAGTCAGTGCCTGAATCTTTATTTTTTAA
25 ATTGAATGTTCCTTAAAGGTTAACATTTCTAAAGCAATATTAAGAAAG
ACTTTAAATGTTATTTTGAAGACTTACGATGCATGTATACAAACGAA
TAGCAGATAATGATGACTAGTTCACACATAAAGTCCTTTTAAGGAGAA
AATCTAAAATGAAAAGTGGATAAACAGAACATTTATAAGTGATCAGTT
AATGCCTAAGAGTGAAAGTAGTTCTATTGACATTCCTCAAGATATTTA

ATATCAACTGCATTATGTATTATGTCTGCTTAAATCATTTAAAAACGG
CAAAGAATTATATAGACTATGAGGTACCTTGCTGTGTAGGAGGATGAA
AGGGGAGTTGATAGTCTCATAAACTAATTTGGCTTCAAGTTTCATGA
ATCTGTAAC TAGAATTTAATTTTCACCCCAATAATGTTCTATATAGCC
5 TTTGCTAAAGAGCAACTAATAAATTAAACCTATTCTTTCAAAAAAAAAA
AAA;

3) AGAAGCAATTTAGGAANCCNACAGNAAANAAATGCTGTTTT
ATAGGAGAGAAAACACGGCACACCAAGGTTAAGTAGTTTGTAGACGAT
10 GTTGAATAGGTTTCAGGTACAGGTCAATGCAGTGATGAGGAAAGCACCT
ANGTATACTTGACAGATAGTCCCCTTTGCTTAACACCCAACTCCTCCA
CCCTGTGCAGTTTNNCTTGTGCCAGTGATCACAGGATTCGCTGAGTGA
ATTACCATAATTGGATTTAATTCACGAAGGGGATGTTTTTC; and

15 4) ATTGATAGAGGCCCTGTTTCATGACATTTTCATGAGTTTCAA
TATGTTGTTTCAGCATGTTGTGAGGTGACTCTCAGCCCCTTTCCCACTG
AGATGGACTGTGGTGATGCTGTGAGGGTGTGACTGACACACCTTCATG
TGCCCAAGCATGGGTTTGATCACAGGTCACATGCAGTTTTTTGGCATAG
TAAATGTATCATTGTTCTTTTCCTCCCTCCTAAAGGAAACAGAGGAAT
20 CCACCTGTATGAGAGTGCCATGTAGGGATAAACTTAAAGGACAGATGA
CACATTGGTCATGTTTCGTGATAAGGAAA.

7. Use of a gene identified by a method according to any one of
Claims 1-6, as a diagnostic marker for the progression and presentation
25 of diabetic nephropathy.

8. Use of a gene identified by a method according to any of Claims 1-6, as an index of disease activity and the rate of progression of diabetic nephropathy.

5 9. Use of a gene identified by a method according to any of Claims 1-6, as a basis for identifying drugs for use in the prevention and/or therapy of diabetic nephropathy.

10 10. A gene containing a sequence selected from any one of sequences 1) - 4) according to Claim 6.

15

ANNE RYAN & CO.

AGENTS FOR THE APPLICANTS

AbstractIdentification of genes having a role in the presentation of diabetic nephropathy.

5

10

15

A method for identifying a gene having a role in the presentation of diabetic nephropathy comprises culturing mesangial cells in the presence of a concentration of glucose sufficient to induce differential expression, especially up-regulation, of a gene susceptible to such differential expression and identifying the gene so induced. The differentially expressed genes can be identified by suppression subtractive hybridisation. The method has resulted in the identification of novel genes which play a role in the presentation of diabetic nephropathy. The genes can be used as diagnostic markers for diabetic nephropathy and as the basis of drug development programmes.

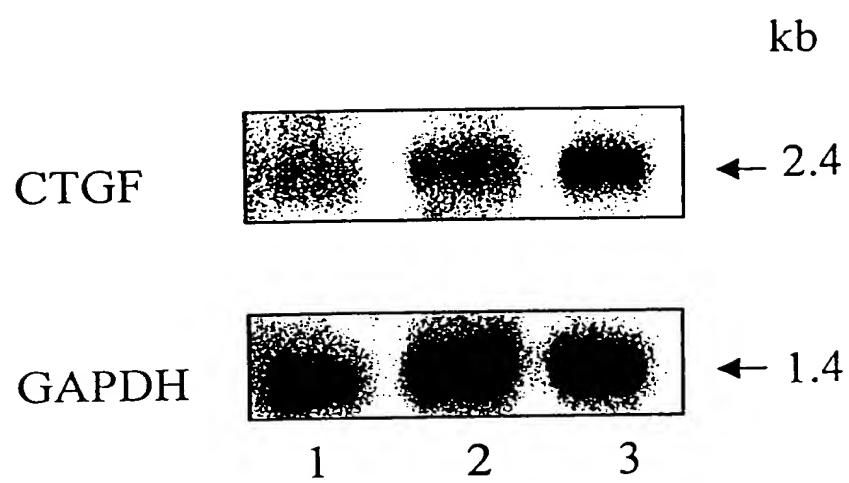


Fig. 1

2/12

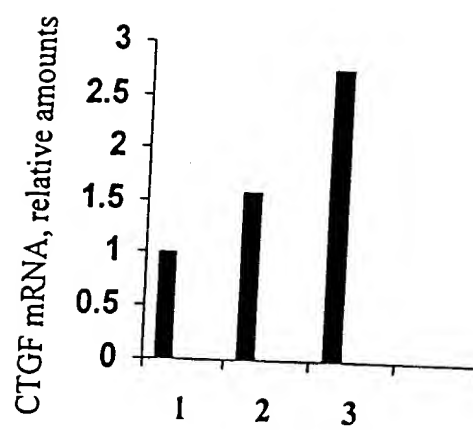


Fig. 2

3/12

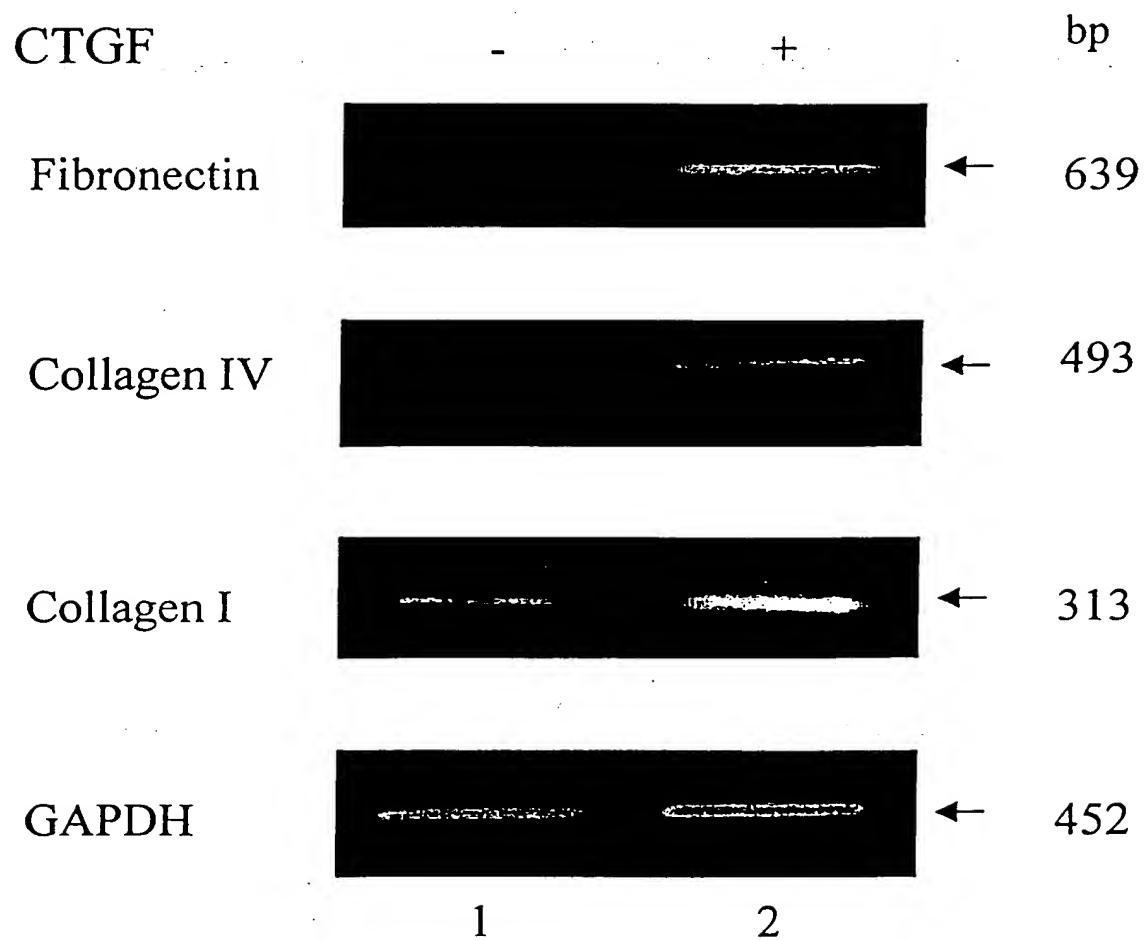


Fig. 3

```

rat: 1      atctccaccgGgttaccaatgacaatacTttctgcagGctggagaagcagagTcgTctc 60
          ||| ||||| ||| ||||| ||| ||||| ||| ||||| ||| |||
mouse: 783   atctccaccgagttaccaatgacaataccttctgcagactggagaagcagagccgcctc 842

    rat: 61   tgcattggtcaggccctgTgaagctgacctAgaggaaaacattaagaagggcaaaaagtgc 120
          ||| ||||| ||| ||||| ||| ||||| ||| ||||| ||| |||
mouse: 843   tgcattggtcaggccctgcgaagctgacctggaggaaaacattaagaagggcaaaaagtgc 902

    rat: 121  atccggacGcctaanaaaTgccaaagcctgtcaagtttgagctttctggctgcaccagtggtg 180
          ||| ||||| ||| ||||| ||| ||||| ||| ||||| ||| |||
mouse: 903   atccggacacctaanaaaatgccaaagcctgtcaagtttgagctttctggctgcaccagtggtg 962

    rat: 181  aaagacCtaacGgggctaagttctgTgggggtgtgcacGgacggccgctgctgcacaccgcac 240
          |||| | ||| ||||| ||| ||||| ||| ||||| ||| |||
mouse: 963   aaagacatacagggctaagttctgTgggggtgtgcacagacggccgctgctgcacaccgcac 1022

    rat: 241  agaaccaccacActgccgggtggagttcaaGtgccccCatggcgaAatcatgaaaaagaac 300
          ||| ||||| ||| ||||| ||| ||||| ||| ||||| ||| |||
mouse: 1023  agaaccaccactctgccagtgagttcaaagtgccccgatggcgagatcatgaaaaagaat 1082

    rat: 301  atgatgttcataagacctgtgcctgccattacaactgtcc 341
          ||| ||||| ||| ||||| ||| ||||| ||| ||||| ||| |||
mouse: 1083  atgatgttcataagacctgtgcctgccattacaactgtcc 112

```

Fig. 4

5/12

rat: 1 ISTRVTNDNTFCRLEKQSRLCMVRPCEADLEENIKKGKKCIRTPKIAKPVKFELSGCTSV 180
mouse: 216 ISTRVTNDNTFCRLEKQSRLCMVRPCEADLEENIKKGKKCIRTPKIAKPVKFELSGCTSV 275

rat: 181 KTYRAKFCGVCTDGRCCCTPHRTTTL PVEFKCPHGEIMKKNNMMFIKTCACHYNC 339
mouse: 276 KTYRAKFCGVCTDGRCCCTPHRTTTL PVEFKCPDGEIMKKNNMMFIKTCACHYNC 328

Fig. 5

6/12

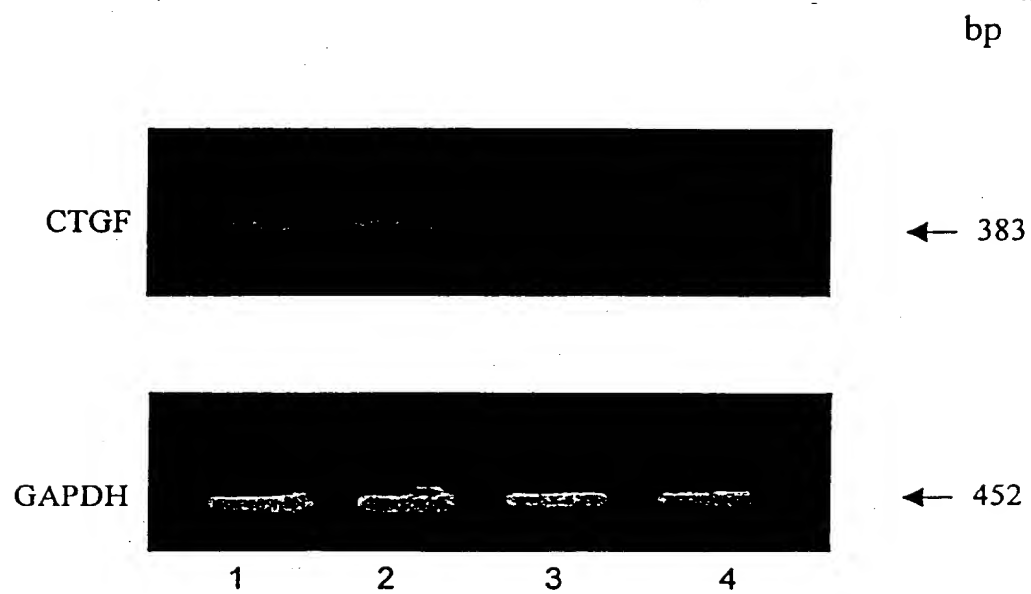


Fig. 6

7/12

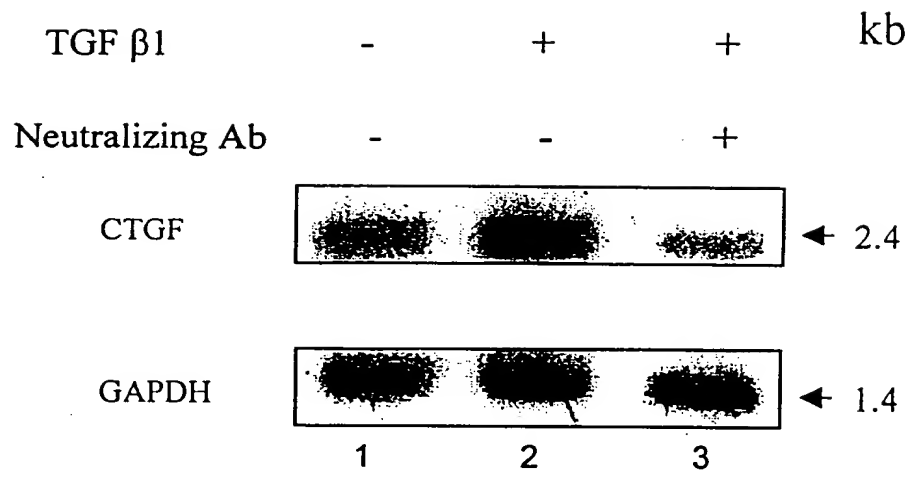


Fig. 7

8/12

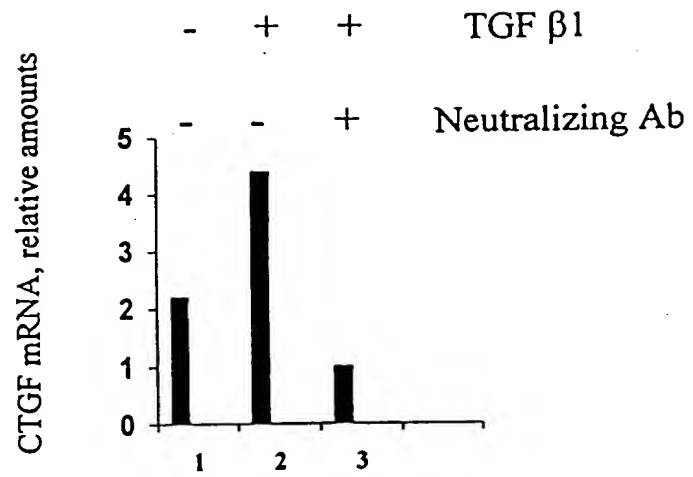


Fig. 8

9/12

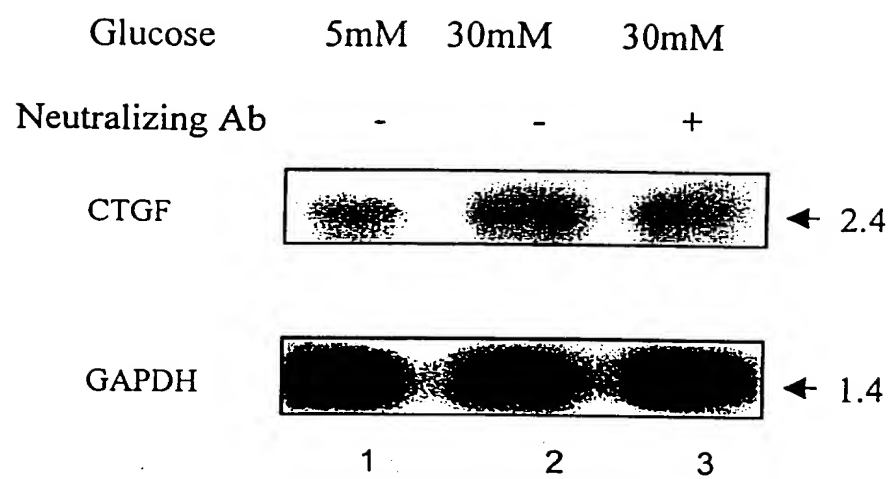


Fig. 9

10/12

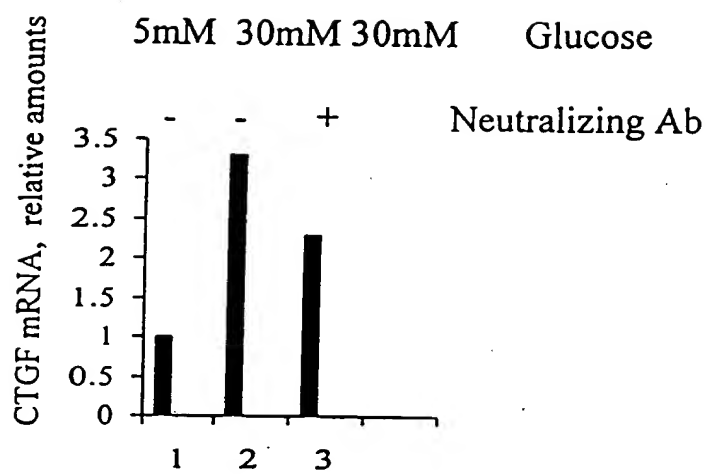


Fig. 10

11/12

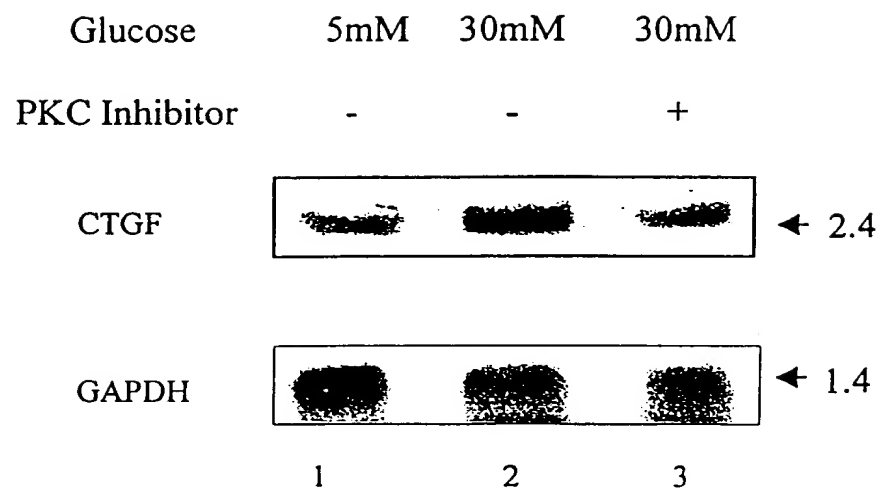


Fig. 11

12/12

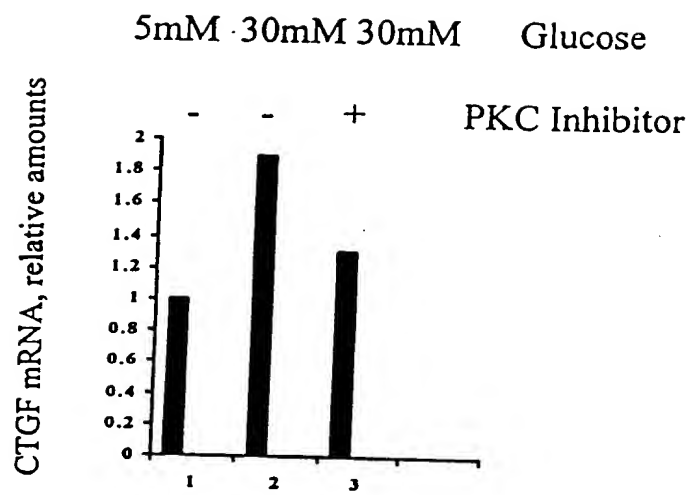


Fig. 12